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TECHNICAL MANUSCRIPT 551

THE IMPORTANCE OF OXIDATION-REDUCTION POTENTIAL LEVELS IN THE GROWTH OF TISSUE CELL LINES, USING EARLE'S L CELLS AS A PROTOTYPE

William F. Daniels
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DEPARTMENT OF THE ARMY
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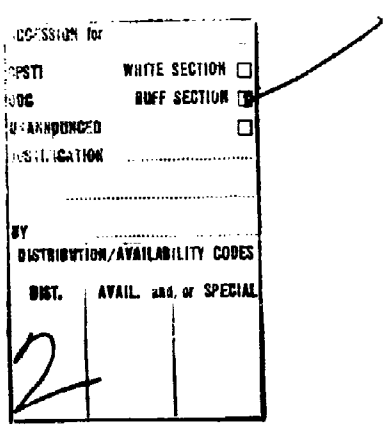

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ABSTRACT

In the course of investigations concerning suspension growth of Earle's L tissue cells, the authors noticed certain erratic growth patterns arising from no recognizable cause. Of environmental factors such as agitator speed, aeration level, temperature, pH, and oxidation-reduction potential level, the latter presented the greatest problem in that least was known about it, and preliminary work showed its potential importance as a most likely cause of erratic growth patterns. Evidence involving oxidation-reduction potential (ORP) as an important factor in environmental control is presented and discussed. In particular, evidence is given showing effects of growth under constant ORP levels, as well as the predetermination of growth caused by initial ORP effects even in the absence of subsequent control. Optimal ranges for the cell-medium system studied are given.

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I. INTRODUCTION*

In the course of our early work on the suspension growth of tissue cells, such as Earle's L, the HeLa, and human amnion, we often noted erratic growth. Aside from the effects of contamination by pleuropneumonia-like (or Mycoplasma species) or other organisms in some instances, we found no accountable factor. We desired, of course, to control all factors in the environment that seemed of possible interest, such as agitator speed, aeration level, temperature, pH, oxidation-reduction potential (ORP) level and dissolved oxygen.

At that time, we felt the effects of oxygen and ORP to be directly related, so that, in the absence of an autoclavable electrode for dissolved oxygen measurement, we turned to ORP measurements. Though many earlier workers had given up this approach, we felt it of interest for two reasons. First, the early equipment was based on essentially a Wheatstone bridge type of measurement that polarizes the electrode by the small current flow that inevitably results from such equipment. Secondly, we noted that some of the best media used at the time contained reductants, such as cysteine and ascorbic acid, which, with the entire media, showed much lower potentials than did much simpler media. We felt that the lower potential could be related to the medium growth potential. An example of the two extremes would be Parker's 1066 medium with cysteine and ascorbic acid, showing an initial +100 mv on the bench (uncorrected for the Ag-AgCl electrode), and an initial ORP of +400 mv for a simple yeast-extract, lactalbumin-hydrolysate, proteose-peptone medium without reductants.

In 1958 Cooper[1] established that, in his work, 7 to 10% oxygen in the gases passing over the surface of the liquid in his culture vessels gave best results. He attributed this success to lower ORP values but did not suggest that ORP values were proportional to dissolved oxygen. Thus, on the assumption that true ORP levels were indeed involved, we measured ORP values in all growing cultures wherever possible.

Finally, based on observations showing a difference in ORP values among different media of different growth potential and others showing differences in ORP patterns of vessels of varying geometric configurations, from Roux bottles to spinner cultures to New Brunswick vessels, we felt two avenues of investigation worthy of attention:

1) Control of the initial ORP of media at time of inoculation (i.e., equilibration).

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2) Control of environmental ORP after inoculation during cell growth. The data and information presented herein are drawn from our experiences with viable cell yields and rates of growth for the LDR variant of Earle's L cell as related to three experimental conditions: (i) no ORP control, (ii) ORP-controlled equilibration of medium, and (iii) ORP-controlled equilibration of medium with subsequent ORP environmental control.

II. MATERIALS AND METHODS

A. CELLS

The cells used in these studies are termed LDR to denote a variant of Earle's strain L cells, which has been carried since 1962 within our laboratory and adapted to suspension growth.

B. INOCULUM

With the exception of that used for the 40-liter fermentor, all inocula were grown in 250-ml centrifuge spinner bottle cultures and used at the 5th day of growth to inoculate all cultures at a level of about 1×10^5 viable cells per ml. The 40-liter fermentor was seeded with the cells from a 7.5-liter New Brunswick fermentor at the 5th day of growth.

C. MEDIUM

The medium described by Rosensteel et al.[2] was used for these studies. Essentially, it is a modified version of Eagle's minimum essential medium for suspension growth with a modified Earle's balanced salt solution, using increased phosphate salts to aid buffering capacity. The medium itself uses the nonessential amino acids with lactalbumin hydrolysate in place of the essential amino acids, and is fortified with cysteine and ascorbic acid. It also is notable that it contains no antibiotics. This medium will support growth of cells for periods up to 8 days without attention, save perhaps occasional pH adjustment. Unless shown otherwise, 10% bovine serum was used in all instances. For some experiments, the freshly prepared medium was poised by one of the following treatments:

1) By incubation at 37 C for 3 days to an undetermined and uncontrolled mv level.

2) By aeration at 37 C to +50 mv.

3) In controlled fermentors, by aeration to a desired mv level, generally +50 mv, with control after inoculation to maintain that level.

In those instances where poising was used, the medium was then frozen to prevent further change until use. The incubated medium was used immediately following incubation.

D. CONTROL EQUIPMENT

The equipment used was that described earlier by Daniels, et al.,[3] for control and recording of pH and ORP. The two systems were identical save in electrodes and material controlled for affecting the pH or ORP. Each consisted of electrodes, amplifiers, indicator-controllers, recorders, and timers to provide delayed action of (i) either acid or base for pH control and (ii) nitrogen-CO_2 or air-CO_2 to manipulate the ORP. Because the equipment is housed in a modular arrangement, it can be used with 1-liter, 4-liter, 7.5-liter New Brunswick, and larger fermentors as desired. Only the recording of ORP was accomplished for the 250-ml centrifuge spinner bottles.

E. FERMENTORS

Four vessels were used: a 250-ml centrifuge spinner bottle for inocula and growth studies, a 1-liter fermentor described earlier by Daniels et al.[3] a 7.5-liter New Brunswick fermentor fitted with electrodes and a heating-cooling coil, and a 40-liter stainless steel agitated bacterial fermentor fitted with electrodes for pH and ORP recording and control.

III. DISCUSSION AND RESULTS

A. ORP AND MEDIUM PROCESSING

We routinely test our medium for sterility by incubating lots comprising 10% of the medium prepared at any one time. Early in our experience we discarded this material routinely as being deficient in nutrients destroyed by incubation at 37 C. Mohberg and Johnson[4] pointed out that ascorbic acid disappears in less than 24 hours when stored at 4 C and thiamine is converted to thiamine disulfide in about 24 hours. Thus, they suggested preservation by removing the oxygen with nitrogen.

Once, when he was using such incubated medium for stock culture because we had been caught short in our regular medium supply, one of our technicians noted better growth. To test this, we compared two groups of media, one incubated for the 3-day test period at 37 C, the other frozen until use. These were made up at one time and represent one batch of medium ingredients; the only difference was the incubation for 3 days. The incubated lot showed 99% probability of significantly better growth. Because we routinely

measured the ORP value of the medium at the time of inoculation, we noticed that 5 pairs of 43 flasks had ORP levels within 3 mv of each other. Additionally, the subsequent growth was remarkably similar. Figure 1 shows two of the five pairs. Although one could conclude that incubation conferred desirable qualities upon the medium, the process did seem ORP-related.

To determine whether ORP levels did change during the incubation, we again prepared an experiment involving three batches of medium; each batch was divided into two parts, one incubated, the other merely frozen until use. The three groups receiving incubation were monitored for ORP changes by incorporating electrodes within the sealed storage vessels. Figure 2 shows the characteristic patterns found within one of the groups. Note the meandering, then the rising to level off with unusual uniformity. The groups of incubated medium did indeed show statistically significant better growth, but the precision of the frozen medium was better.

Again classifying the data into four groups according to ORP at the time of inoculation, one could note that the average growth rates (here defined as the increase in viable cells per ml per day between the 1st and 4th days) and the average peak growths (defined as the maximum viable cells per ml achieved) of the groups were significantly different and seemingly related to ORP levels at time of inoculation. With this in mind, we desired to establish whether some effort to control the ORP levels in simple spinner vessels, at least at the time of inoculation, might be worthwhile, even in the absence of control during cell growth. Of course, the ORP level would be influenced by the diffusion of oxygen into the vessel through the filter vents normally employed.

Both to gain information on the above points and to compare growth in the same type of medium with 5% versus 10% serum in the hope of reducing serum levels, we set up experiments in which all media were poised by sparging with air plus 5% CO_2 until the ORP level was +50 mv. This was accomplished in a modified New Brunswick fermentor in which controlled sparging could be achieved. The medium was then placed in bottles, stoppered, and frozen or incubated until inoculation within the centrifuge spinner bottle.

Figure 3 shows the average curves for each of the four treatments together with a data curve from earlier work representing unpoised incubated medium with 10% serum for comparison. Medium that was poised and then frozen exhibited the best growth rates and peaks at both serum levels. Statistical analysis confirmed that better reproducibility also accrued with poised and frozen medium at both serum levels but was best at the 10% level. When the data from the earlier work with unpoised incubated medium containing 10% serum were plotted for comparison, the peak growth was not statistically different from that obtained with poised and frozen medium containing 10% serum, but the growth rate of the former was not as great as that of the latter. The precision was much inferior with unpoised medium, in computing both growth rates and peaks. Thus, one may say the precision of growth achieved is a very great benefit to be derived from poising medium before use. Certainly, one can disregard incubation after poising.

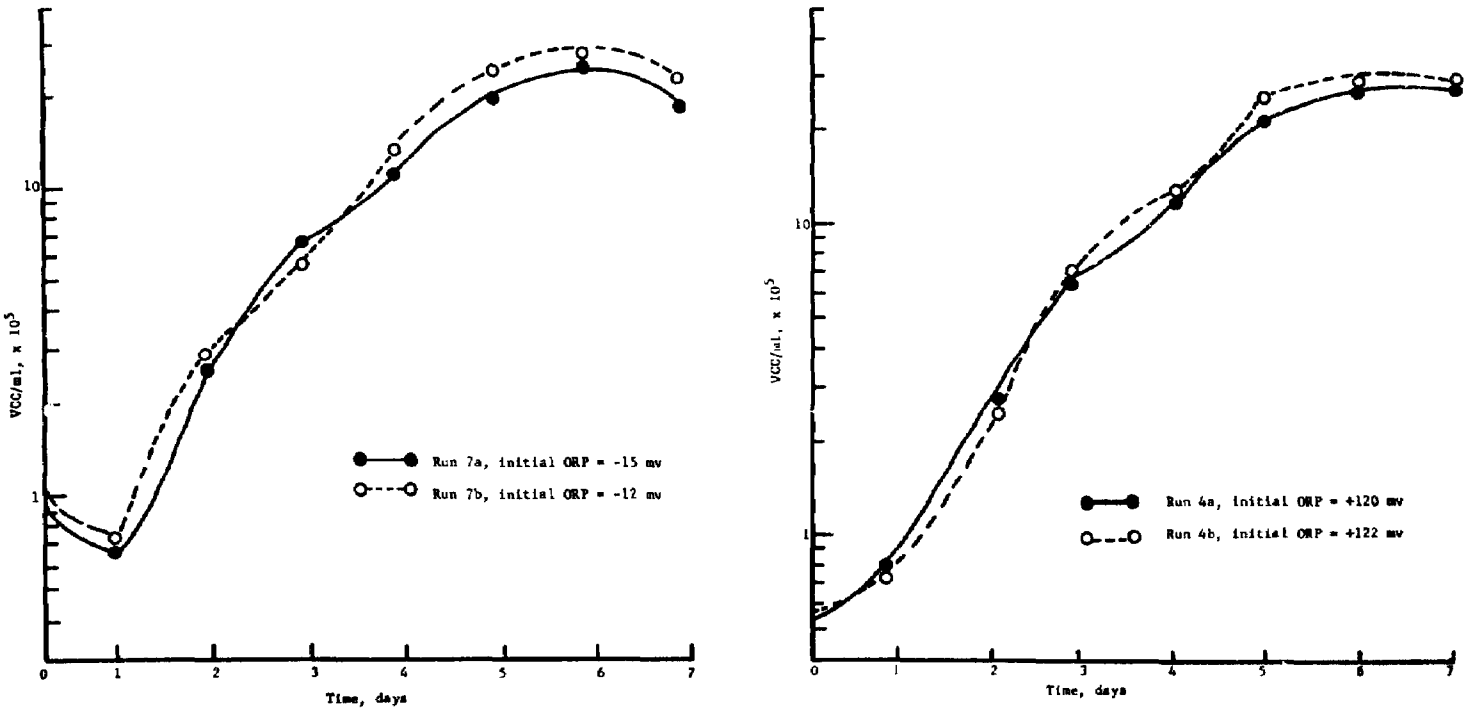

FIGURE 1. Paired Growth Curves of Spinner Cultures Grown in Medium Lots with Initial ORP Levels within 3 mv of Each Other. The medium lots for the paired curves on the left were not incubated prior to use, and the growth curves exhibit a marked lag phase; the lots for the curves on the right were incubated for 3 days at 37 C, and no lag phase is evident.

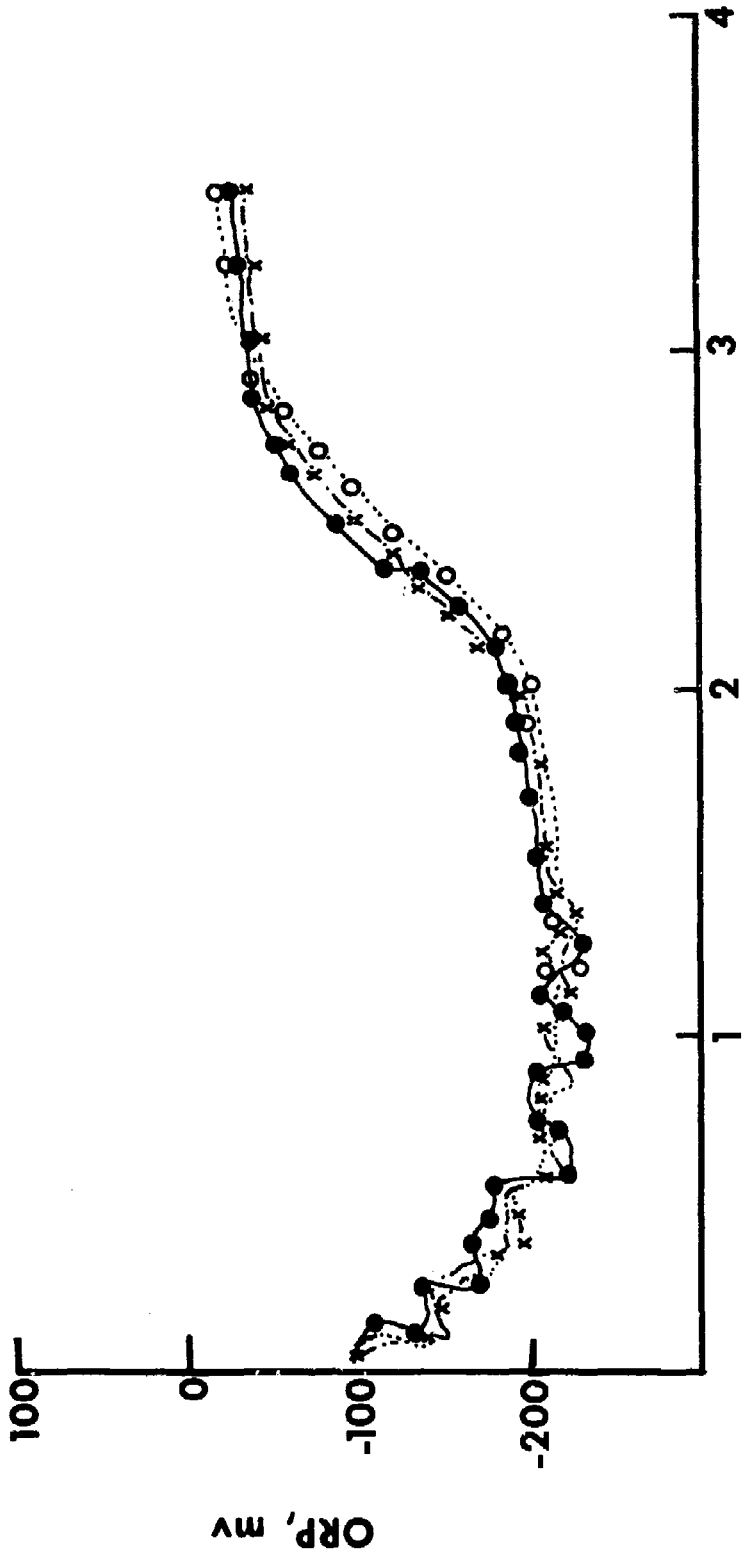

FIGURE 2. ORP Patterns Generated Without Aeration During Incubation of Medium in Three Centrifuge Spinner Bottles.

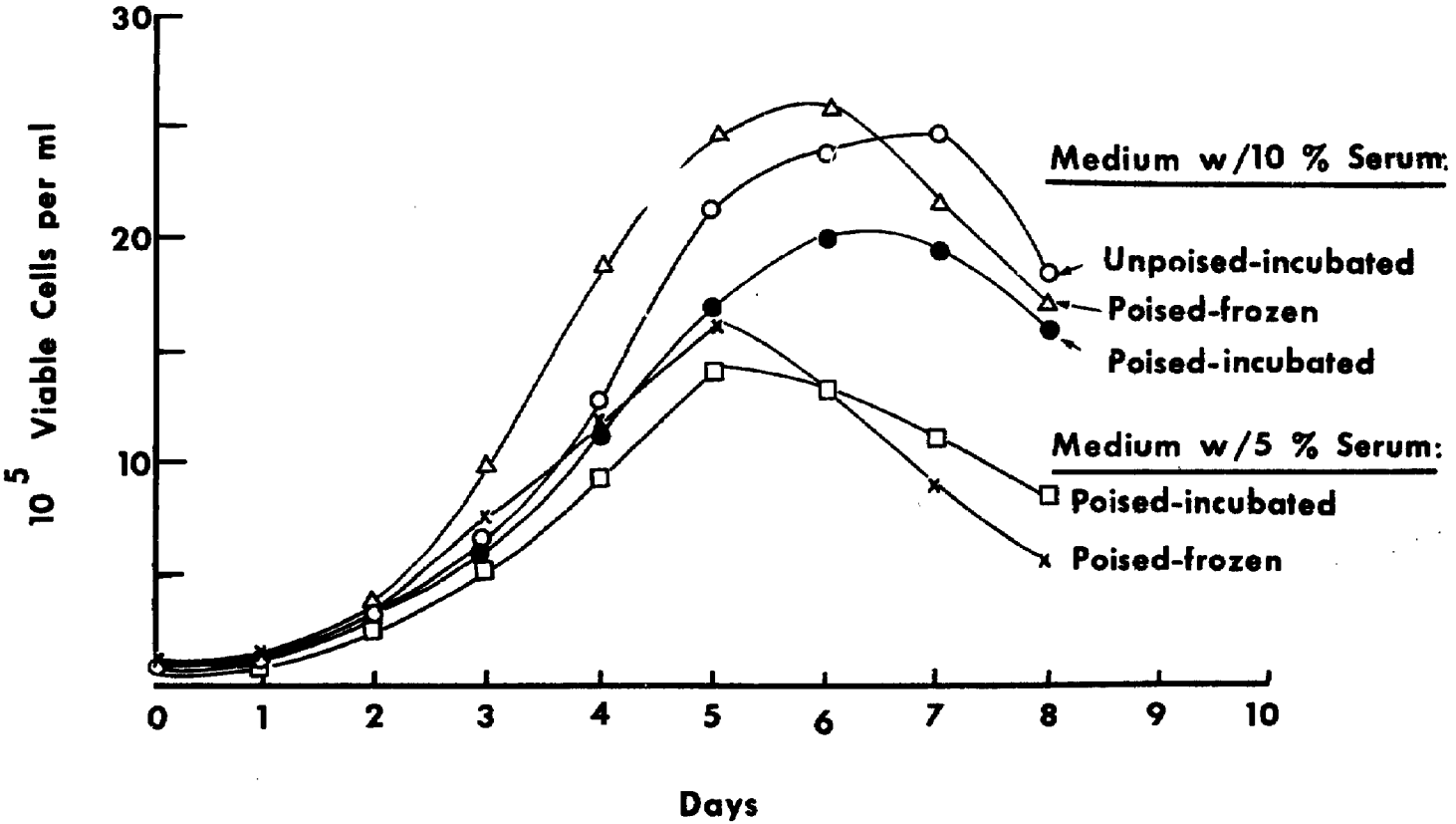

FIGURE 3. A Comparison of the Average Growth Curves for Different Medium Treatments, Depicting Relative Growth Rates and Growth Peaks.

B. ORP AND CONTROL IN FERMENTORS

The first observations leading to control of ORP in fermentors were made in Roux bottles.[5] These showed a uniform drop from about +80 mv to 0 during the first 3 days. The initial level was restored by the feeding on the 3rd day, but it again dropped to -20 mv by the 5th day. The initial point again was restored by the feeding on the 5th day but dropped to -45 mv by harvest on the 7th day. During this period, only a ninefold increase in cells was noted.

When a 7.5-liter New Brunswick fermentor was operated with a gas overlay but no sparging, the ORP dropped from +100 to -120 by the 7th day. Although the initial overlay mixture contained 7% oxygen, increasing the percentage to ordinary air plus 5% CO_2 did not change the course of the dropping ORP.

In contrast, the simple 250-ml centrifuge spinner was not gassed with an overlay mixture, but simply vented to the atmosphere through a cotton filter. The ORP levels rose from about +20 to +90 mv during the first 24 hours and remained level till the 3rd day, when it dropped to about +10 mv in 4½ days and then rose to about +100 mv at the end of 6½ days.

The pattern obtained in a 1-liter vessel reported elsewhere[3] showed a somewhat similar pattern in spite of being agitated and gassed with an overlay of 7% oxygen plus 5% CO_2. For example, from the initial level of about +30 mv, the ORP rose to +160 by the 2nd day, remained more or less level till the 4th day, dropped to -10 by the 8th day, then rose to +110 by the 9th day. Growth was very good, albeit slow. The 7% oxygen level gave the best results when only overlay gassing was used.

To recapitulate these experiences, two basic types of curves were obtained for two types of cultures: monolayers in sealed flasks and suspension cultures. The latter showed two types of curves, one with a continued dropping similar to that of the sealed monolayer flask and the other with an initial rise, leveling off, dropping to a low, and then rising to a final high with cessation of growth. This second type was also seen in cultures with no aeration save venting and also in cultures with a gas overlay. These findings led us to explore the possibility of controlling ORP at a fixed level from start to finish, with the realization that perhaps varying values at different points along the growth curve might be more beneficial.

Figure 4 shows a run made with the 7½-liter New Brunswick fermentor with the control ORP held at about +50 mv. Although the control shows faster growth at first, the fermentor eventually approaches 25% greater cell growth. The control consisted of the inoculated medium at the same level in a 250-ml centrifuge bottle spinner culture. The viability of the control remained higher toward the end of the growth cycle in comparison to the fermentor, a fact that suggests environmental selection to account for the increased cell numbers at the same time in the fermentor.

Noteworthy on this figure is the curve along the X axis at the bottom. Here is represented the nitrogen plus 5% CO_2 or air plus 5% CO_2 needed to maintain the ORP at +50 mv. The greatest need is at a count about 1.4 x 10^6 viable cells/ml, a figure contrasting with the peak of about 2.2 x 10^6. This suggests fundamental changes in the metabolism about 28 hours before the peak growth is achieved. The causes are the subject of other investigations.

With the success of a controlled run at +50 mv, we next turned to the question of whether or not optima exist for ORP under controlled conditions. Certainly interaction among factors such as pH, ORP, agitation, and nutrition could play an important part in achieving optimal growth conditions. For simplicity at the outset, we kept the medium as constant and complete as we knew how to make it. Figure 5 shows some typical runs under such conditions with ORP levels kept constant at levels of -50, +50, 100, and 150 mv. The ORP values shown refer to the Ag-AgCl electrode and are not corrected to the hydrogen electrode. The figure shows that best growth occurred at +100 mv. Theoretically, a better choice could be +75 mv or perhaps +120 to +125 mv, because, according to Hongo,[8] cytochrome systems are active in those ranges. The cytochrome systems are those enzyme systems important in cell respiration. Nominally, one would think that transfer through the cell wall would be slow and controlling, but some workers have suggested that transfer is very fast. Therefore, if this is true, an optimal value close to that of cytochromes within the cell could exist. With the equipment currently in use, this would be difficult to demonstrate.

From 16 experiments made with minimal equipment and electrode difficulties, the curves shown in Figure 6 were derived. Here the data suggest a broad upper plateau of good peak growths from 0 to +100 mv. The rate of growth, however, is different. For engineering purposes, the rates presented were calculated from the 1st through the 4th day arbitrarily and represent the average increase in viable cells per ml per day. Thus, one could select +100 mv as giving good peak growth with best rate of production.

More detailed data on ORP and cell growth in larger stainless steel vessels is presented elsewhere.[7] However, some data, taken with a typical stainless steel agitated bacterial fermentor fitted with pH and ORP electrodes for automated pH and manual ORP control, is of interest here. Table 1 shows the data resulting from control samples of the media at different steps in the operation. The media inoculated and incubated after sterilization in our laboratory and in another laboratory both showed no growth at ORP values of about -60 mv in spinner bottles. Aeration must have taken place at least to some extent in the filtration steps. On the other hand, sparging with air and lowering the pH slightly produces excellent growth. Because reports exist showing cell growth from pH 6.7 to 7.4, one must conclude that growth was suppressed by the lower initial ORP.

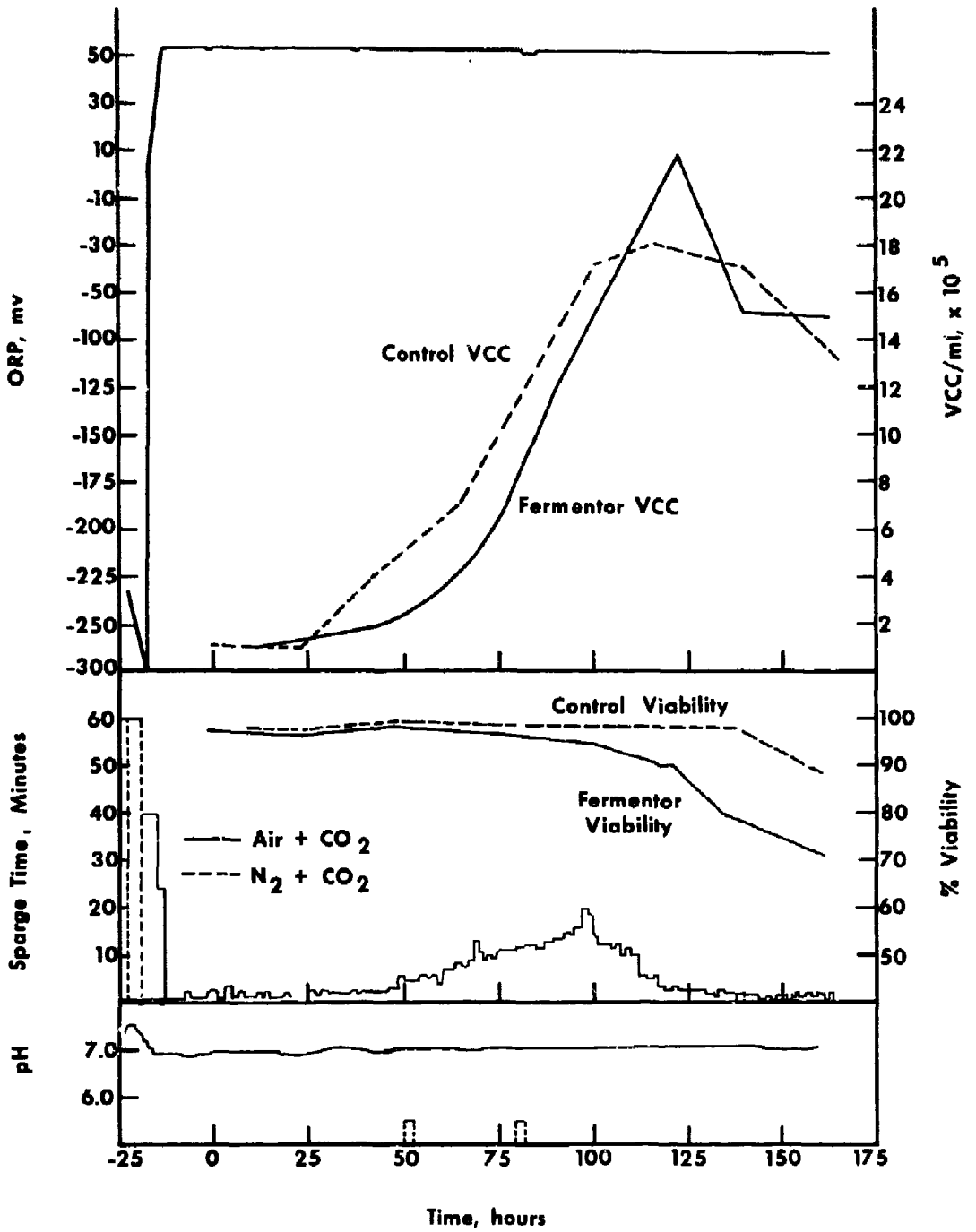

FIGURE 4. Effect of Controlled pH and ORP on the Growth of L Cells in a 7.5-Liter New Brunswick Fermentor.

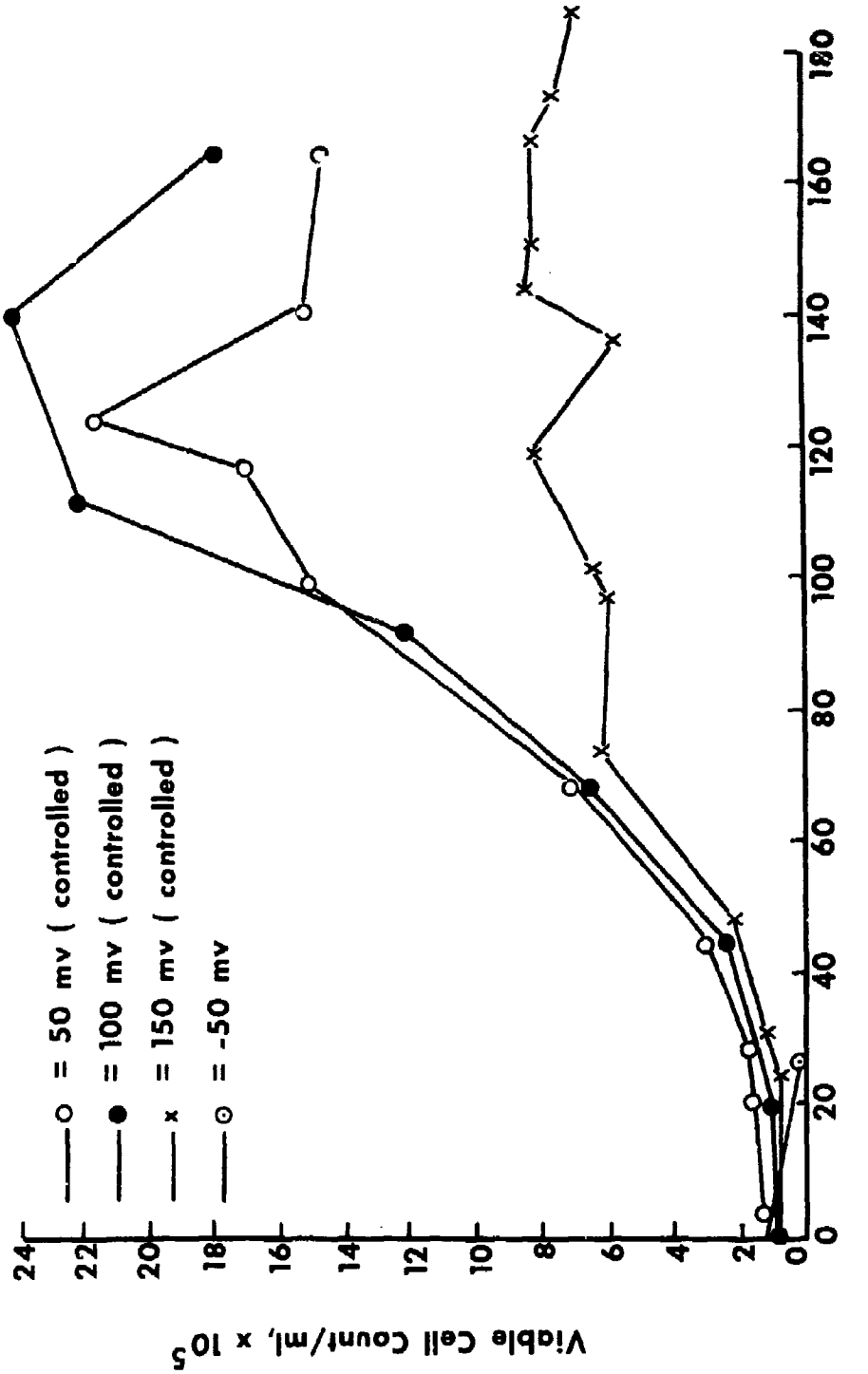

FIGURE 5. Typical Tissue Culture Growth Curves at Various Constant ORP Levels.

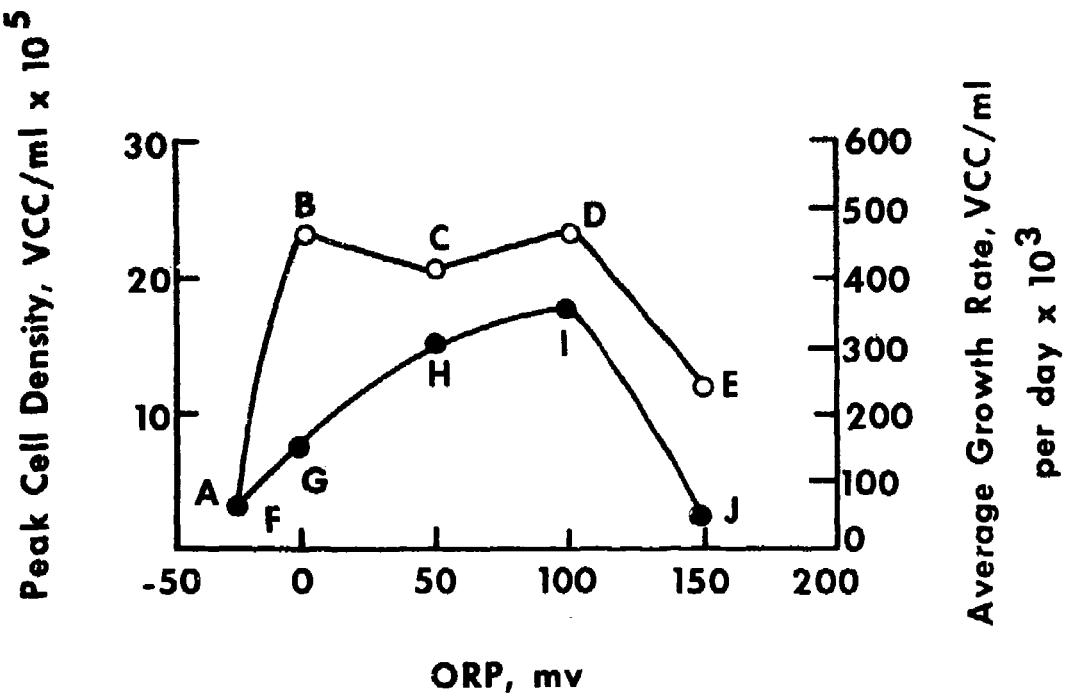

FIGURE 6. Peak Cell Density (Curve A-E) and Average Growth Rate (Curve F-J) Versus Oxidation-Reduction Potential.

TABLE 1. COMPARISON OF THE GROWTH OF L CELLS IN A 40-LITER NEW BRUNSWICK FERMENTOR AND CONTROL SPINNER BOTTLES

Fermentor	Medium Conditions	pH	ORP, mv	Viable Cell Count/ml, x 10^5
40 Liter	Filtered in Plant; pH and ORP adjusted	6.82	+50	37.5
Spinner Bottle	Filtered in Plant	7.0	-62	0
Spinner Bottle	Filtered in Lab	7.15	-59	0
Spinner Bottle	Drained from 40-Liter Fermentor which had pH and ORP Adjusted	6.82	+50	32.5

Figure 7 shows data from the fermentor run. The growth was excellent, but the rate was somewhat slower than that of other runs in which the peak occurred between the 5th and 6th days. In this run, the ORP was manually controlled, and was relatively steady until the 40th hour, at which time it slowly dropped to the low of about 0 then rose slowly back to the control level around +50 mv. The mixing rotameters were not sufficiently large to permit metering 5% CO_2 at the fastest rate needed to maintain the ORP; therefore, the system slowly fell behind. Other experiments suggest that too rapid a shift in ORP or pH will affect the cell growth by slowing the growth rate till the equilibria are restored. The slow drop and subsequent increase were not too abrupt for this system. The actual ORP levels in terms of the saturated Ag-AgCl electrode probably were between a low of +25 mv and a high of 85 mv. Thus, from the earlier data, one would conclude that growth was permissible in this range though not necessarily at the fastest rate.

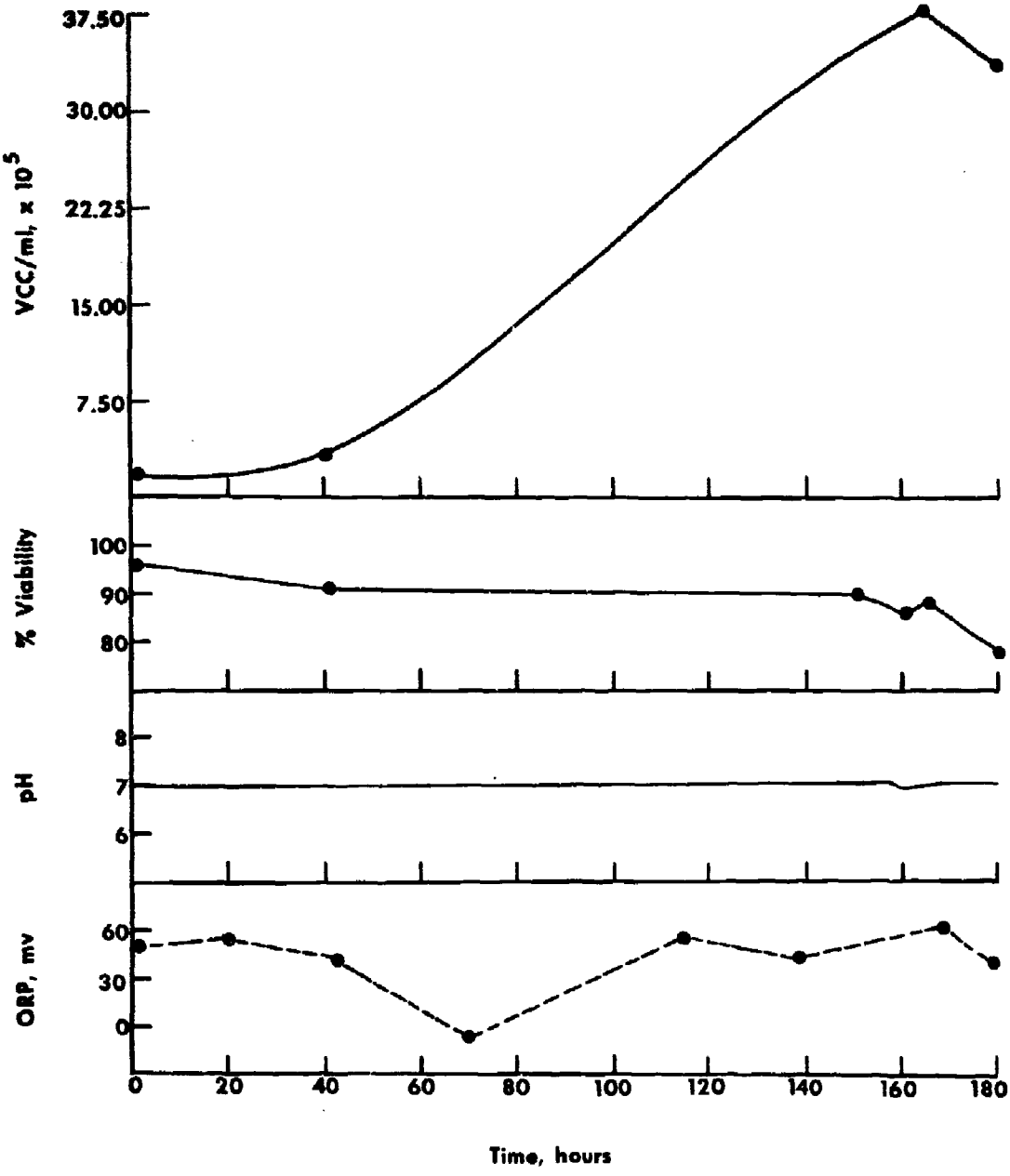

FIGURE 7. Submerged Culture Growth of L Cells in a 30-Liter Fermentor.

IV. CONCLUSION

The present relationship between ORP levels and cell growth in our laboratory may be summarized in two ways.

First, ORP levels of medium at the moment of inoculation are related to subsequent growth performance. Thus, an equilibration of our medium occurs after mixing, and this definitely is related to growth characteristics. This equilibration is ORP-related. Evidence shows that this equilibration can be affected at least to some extent by poising with sparging, a process that affords better precision in growth characteristics compared with non-treatment, even when control after inoculation cannot be achieved.

Secondly, in controlled fermentors, an optimum ORP range exists for maximum cell growth and rate of cell growth. The range seems more critical for growth rates than for maximum growth. Controlled ORP levels in growth of cells offer a means of affording aeration response in growth without undue regard for vessel geometry.

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13. ABSTRACT

In the course of investigations concerning suspension growth of Earle's L tissue cells, the authors noticed certain erratic growth patterns arising from no recognizable cause. Of environmental factors such as agitator speed, aeration level, temperature, pH, and oxidation-reduction potential level, the latter presented the greatest problem in that least was known about it, and preliminary work showed its potential importance as a most likely cause of erratic growth patterns. Evidence involving oxidation-reduction potential (ORP) as an important factor in environmental control is presented and discussed. In particular, evidence is given showing effects of growth under constant ORP levels, as well as the predetermination of growth caused by initial ORP effects even in the absence of subsequent control. Optimal ranges for the cell-medium system studied are given.

14. Key Words

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Earle's L cells
Cell growth patterns
Tissue culture
Medium incubation
Growth pattern prediction

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